AVIAN DISEASES 54:1002–1006, 2010

Comparison of *Eimeria* Species Distribution and Salinomycin Resistance in Commercial Broiler Operations Utilizing Different Coccidiosis Control Strategies

M. Jenkins,[AD] S. Klopp,[B] D. Ritter,[C] K. Miska,[A] and R. Fetterer[A]

[A]Animal Parasitic Diseases Laboratory, Agricultural Research Service, USDA, Beltsville, MD 20705
[B]Townsends Poultry, Georgetown, DE 19947
[C]Mountaire Farms, Inc., Millsboro, DE 19966

Received 15 November 2009; Accepted and published ahead of print 21 April 2010

SUMMARY. The purpose of the present study was to evaluate the species composition and salinomycin sensitivity of *Eimeria* oocysts isolated from commercial broiler farms that differed by means of coccidiosis control (anticoccidial drugs [ACD] *vs.* live oocyst vaccines [VAC]). A comparison of *Eimeria* species composition and salinomycin sensitivity was also made before and after a producer switched from salinomycin to live oocyst vaccines. In general, no significant difference was observed in the concentration of *Eimeria* spp. oocysts in litter from VAC-utilizing farms compared to litter from ACD-utilizing farms. Application of PCR-based methods to detect coccidia found that *Eimeria* species distribution in litter from VAC operations more closely resembled the species composition in the live oocyst vaccines. Drug sensitivity testing found that *Eimeria* oocysts from VAC operations displayed greater salinomycin sensitivity as measured by weight gain and feed conversion efficiency compared to oocysts from ACD farms. These findings provide additional evidence for the usefulness of live oocyst vaccines to restore ionophore sensitivity in poultry operations that contain an ionophore-resistant population of *Eimeria* spp. oocysts.

RESUMEN. Comparación de la distribución de las especies de *Eimeria* y de la resistencia a la salinomicina en operaciones comerciales de pollo engorde que utilizan diferentes estrategias para el control de la coccidiosis.

El propósito del presente estudio fue evaluar la composición de las especies y la sensibilidad a la salinomicina de los ooquistes de *Eimeria* aisladas de granjas comerciales de pollos de engorde que utilizaban diferentes medidas de control contra la coccidiosis (fármacos anticoccidiales en comparación con vacunas con ooquistes vivos). Se realizaron comparaciones de la composición de las especies de *Eimeria* y de la sensibilidad a la salinomicina antes y después de que el productor cambiara de la aplicación de la salinomicina al uso de vacunas con ooquistes vivos. En general, no se observaron diferencias significativas en la concentración de ooquistes de *Eimeria* spp en la cama de las granjas que utilizaban la vacuna en comparación con la cama de las granjas en donde se utilizaban drogas anticoccidiales. Mediante la aplicación de métodos basados en la reacción en cadena de la polimerasa para la detección de coccidias, se encontró que las especies de *Eimeria* en la cama de las operaciones donde se aplicaba la vacuna eran muy semejantes a la composición de las especies que se encuentran en las vacunas con ooquistes vivos. Las pruebas para la sensibilidad a las drogas mostraron que los ooquistes de *Eimeria* de las operaciones donde se aplicaba la vacunación eran más sensibles a la salinomicina, lo cual fue determinado por el aumento de peso y por la eficiencia de la conversión alimenticia en comparación con los ooquistes provenientes de las granjas donde se aplicaban drogas anticoccidiales. Estos resultados proporcionan evidencia adicional sobre la utilidad de las vacunas de ooquistes vivos para restaurar la sensibilidad a los ionóforos en las operaciones avícolas que contienen una población de ooquistes de *Eimeria* resistente a estos compuestos.

Key words: *Eimeria*, PCR, vaccine, anticoccidial drugs

Abbreviations: ACD = anticoccidial drug; AdFCR = adjusted feed conversion ratio; DST = drug sensitivity trial; FCR = feed conversion ratio; ITS1 = internal transcribed spacer 1; UIC = uninfected control; VAC = live oocyst vaccine; WG = weight gain

Coccidiosis is an intestinal disease caused by protozoa in the genus *Eimeria*. Outbreaks of avian coccidiosis are marked by poor weight gain (WG) and feed utilization due in part to disruption of epithelial cells lining the intestine. Coccidiosis control has generally involved medication of feed with anticoccidial drugs (ACDs), such as ionophores and synthetic chemicals (1). However, drug resistance in coccidia and a growing consumer desire for broilers raised ''antibiotic-free'' has led to commercial vaccines based on administration of low doses of virulent (e.g., Coccivac, Advent, Immucox, Inovocox) or attenuated (e.g., Paracox, HatchPak CocciIII) *Eimeria* spp. oocysts (2,21,23,24). Virtually all *Eimeria* oocyst vaccines contain *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* based on the assumption that these species are both pathogenic and prevalent on poultry farms. Accurate identification of species of *Eimeria* that are present may be helpful in selecting a vaccine that may be most efficacious because there is negligible cross-protection between *Eimeria* species (e.g., vaccination with *E. acervulina* does not protect against *E. tenella* infection). Unfortunately, most *Eimeria* oocysts that infect chickens are similar in size and morphology and thus, aside from *E. maxima*, cannot be reliably distinguished from each other using microscopy. A number of molecular-based methods, such as PCR, have been developed that can identify each of seven *Eimeria* species that infect chickens (5,6,12,14,20). Several of these methods have been applied to the study of coccidiosis epidemiology, and have revealed the diverse nature of *Eimeria* populations in commercial broiler farms (7,10,11,15,16,22). Knowing which species of *Eimeria* are present in a particular operation will allow for more precise formulation of live oocyst vaccines (VACs), and may eventually lead to identification of immunovariant, highly virulent, or drug-resistant strains of *Eimeria* in the future. The purpose of the present study was to compare *Eimeria* species composition and salinomycin resistance between commercial poultry operations that differed by means of coccidiosis control (ACD *vs.* VAC).

[D]Corresponding author. E-mail: mark.jenkins@ars.usda.gov

Table 1. Concentration and species composition of *Eimeria* oocysts recovered from litter in commercial broiler operations that differed by means of coccidiosis control (ACD *vs.* VAC).

Geographic location/coccidiosis control	Oocyst concentration (oocysts/g)[A]	*Eimeria* species[B]				
		Ea	Emax	Emit	Ep	Et
Arkansas/drug[C]	$1.2 \times 10^3 \pm 1.6 \times 10^3$	5/5	4/5	0/5	1/5	3/5
Arkansas/vaccine 1[D]	$3.9 \times 10^4 \pm 4.9 \times 10^4$	5/5	5/5	0/5	4/5	5/5
North Carolina/drug[C]	$6.5 \times 10^4 \pm 3.9 \times 10^4$	5/5	5/5	0/5	1/5	0/5
North Carolina/vaccine 1[D]	$6.6 \times 10^4 \pm 3.8 \times 10^4$	5/5	5/5	5/5	0/5	5/5
Delmarva/drug[E]	$1.4 \times 10^4 \pm 1.8 \times 10^4$	8/8	5/8	0/8	1/8	0/8
Delmarva/vaccine1[D]	$3.5 \times 10^4 \pm 1.5 \times 10^4$	3/3	3/3	0/3	0/3	2/3
Delmarva/vaccine2[D]	$5.4 \times 10^4 \pm 2.6 \times 10^4$	5/5	3/5	0/5	0/5	2/5

[A]Values are given as mean ± SD.
[B]Ea, *E. acervulina*; Emax, *E. maxima*; Emit, *E. mitis*; Ep, *E. praecox*; Et, *E. tenella*.
[C]Rotation between salinomycin alone: starter and grower and chemical/ionophore: nicarbazin, starter; monensin, grower.
[D]Vaccine1, Coccivac B; Vaccine 2, Inovocox.
[E]Salinomycin: starter and grower.

MATERIALS AND METHODS

Isolation of *Eimeria* oocysts from litter. Litter samples were collected from broiler houses at multiple locations in North Carolina and Arkansas. Between 15 and 20 fecal samples were collected from random locations in each poultry house. The poultry operations were differentiated by means of coccidiosis control (i.e., ACD or VAC). ACD operations used a rotation between salinomycin alone (Biocox™, 60g/ton, starter and grower; Alpharma, Fort Lee, NJ) and chemical/ionophore (nicarbazin, starter; monensin, grower), with roxarsone (45.4 g/ton) included in both formulations. VAC operations used Coccivac B™ (Schering-Plough, Kenilworth, NJ) delivered to day-old chicks at hatch via spray cabinet. In addition, litter samples were collected from random locations in broiler houses from the Delmarva region before and after a grower switched from salinomycin (Biocox, 60 g/ton) and roxarsone (45.4 g/ton) to VAC. The vaccines used were either Coccivac B (Schering-Plough) or Inovocox™ (Pfizer, Groton, CT). *Eimeria* oocysts were isolated from poultry litter using standard procedures (11). In brief, litter from each house was homogenized and multiple fecal samples (total weight = 10 g) from the homogenate were transferred to a 50-ml polypropylene test tube, saturated with water, and dispersed by vortexing. The fecal material was placed on a rotating shaker overnight to ensure separation of *Eimeria* oocysts from fecal debris. The tubes were incubated upright for 3 min, and the supernatant was collected and subjected to sucrose flotation using standard techniques (4). Oocyst concentrations were estimated by counting *Eimeria* isolated after sucrose flotation using a hemacytometer.

Propagation of *Eimeria* oocysts. *Eimeria* oocysts isolated from litter obtained from either ACD-utilizing or VAC-utilizing farms were pooled and then inoculated *per os* into susceptible chickens to provide sufficient oocysts for drug sensitivity trials. Feces were collected from days 3–7 postinoculation and processed for total *Eimeria* oocysts using standard procedures (19).

DNA extraction and species determination of *Eimeria* oocysts. *Eimeria* oocysts were treated with 100% household bleach (2.5% sodium chlorite) for 15 min at room temperature on a rotating shaker. Residual bleach was removed by repeated (5×) suspension of the oocysts in water and centrifugation at 2500 × *g* for 10 min. *Eimeria* DNA was prepared by suspending the oocysts in buffer AL (Qiagen, Inc., Valencia, CA) followed by disruption of the oocysts by two 2-min extractions with 0.5-mm sterile glass beads on a Mini-Bead Beater (Bio-Spec Products, Inc., Bartlesville, OK). The DNA isolated from oocysts was purified using a mini-spin column and instructions provided by the manufacturer (Qiagen). After spin-column elution, the DNA was further concentrated by ethanol precipitation, allowed to air-dry, and suspended in 20 μl 0.1× TE (1 mM Tris-HCl, pH 6.8, 0.1 mM EDTA). DNA concentration and purity was estimated by absorbance reading at OD_{260}/OD_{280}. *Eimeria* species determination was conducted using primers and internal standards specific for *E. acervulina*, *Eimeria brunetti*, *E. maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, or *E. tenella* in internal transcribed spacer 1 (ITS1) rDNA PCR as described (10).

Salinomycin sensitivity testing. *Eimeria* oocysts derived from ACD- or VAC-utilizing farms were assayed in drug sensitivity trials (DSTs) using standard procedures (3,8). In the DST studies, broiler chickens (Hubbard × Cobb [Longeneckers Hatchery, Elizabethtown, PA], 3 subgroups/treatment, $n = 3$ chickens/subgroup, total 9 chickens/treatment) were either fed a nonmedicated standard poultry ration (24% protein), or feed medicated with salinomycin (Biocox, 60g/ton) using standard procedures (3,8). Experimental groups were infected with 2×10^5 *Eimeria* oocysts derived from ACD- or VAC-using poultry operations, and given either nonmedicated or salinomycin (Biocox, 60g/ton)-medicated feed. Control groups consisted of broilers not infected with ACD- or VAC-derived *Eimeria* oocysts. All chickens were weighed on day of challenge infection and 7 days after challenge in order to calculate WG during the infection period. Feed consumption was calculated by weighing feed just prior to challenge infection and at termination. Feed conversion ratio (FCR) was calculated for each subgroup by dividing total feed consumed by total WG. Adjusted FCR using the standard conversion of 5 points WG for 1 point FCR was calculated using the following formula:

$$\text{Adjusted FCR} = \text{FCR} + ((WG_{UIC} - WG_{EXP})/500),$$

where WG_{UIC} equals average WG of respective uninfected control and WG_{EXP} equals average WG of experimental group.

Statistical analysis. Mean WG and adjusted FCR were compared between treatment groups using ANOVA and differences between means detected using Duncan's multirange test (SAS Institute, Inc., Cary, NC). Statistical significance between groups was considered if $P \leq 0.05$. WG improvement between groups fed nonmedicated or salinomycin-medicated feed was calculated as a percentage of the respective noninfected control as the baseline WG using the following formula:

$$\text{WG improvement (\%)} = (\text{WG salinomycin-medicated + oocyst infected/WG salinomycin-medicated noninfected}) \div (\text{WG nonmedicated + oocyst infected/WG nonmedicated noninfected}).$$

Mean oocyst concentrations were compared between ACD- and VAC-using poultry operations by ANOVA and differences between means detected using the Tukey-Kramer multiple comparisons test employing GraphPad Instat software (GraphPad Software, Inc., San Diego CA). Statistical significance between groups was considered if $P \leq 0.05$.

RESULTS

Comparison of *Eimeria* species distribution between ACD- and VAC-utilizing broiler operations. Average *Eimeria* oocyst concentrations in litter were between 1.2×10^3 and 6.5×10^4

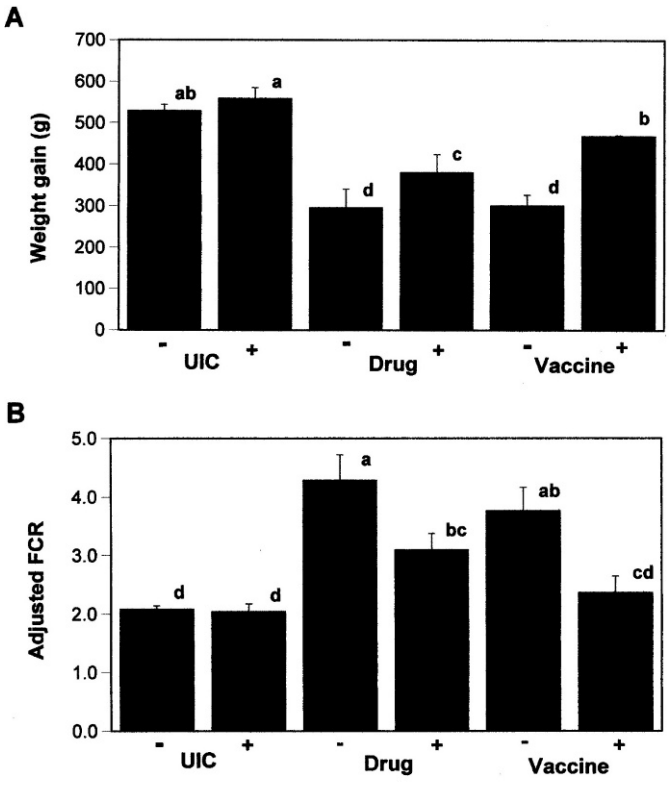

Fig. 1. Protective effect of salinomycin against (A) decreased WG and adjusted feed conversion efficiency (B) associated with coccidiosis in broilers infected with *Eimeria* oocysts isolated from ACD-utilizing or live VAC-utilizing (Coccivac B) poultry operations in Arkansas. UIC, uninfected controls; −, groups given non-medicated feed; +, groups given salinomycin (Biocox, 60g/ton)-medicated feed. Adjusted FCR, adjusted feed conversion ratio. Groups sharing a letter do not have a significant difference ($P > 0.05$) in WG or adjusted FCR from each other.

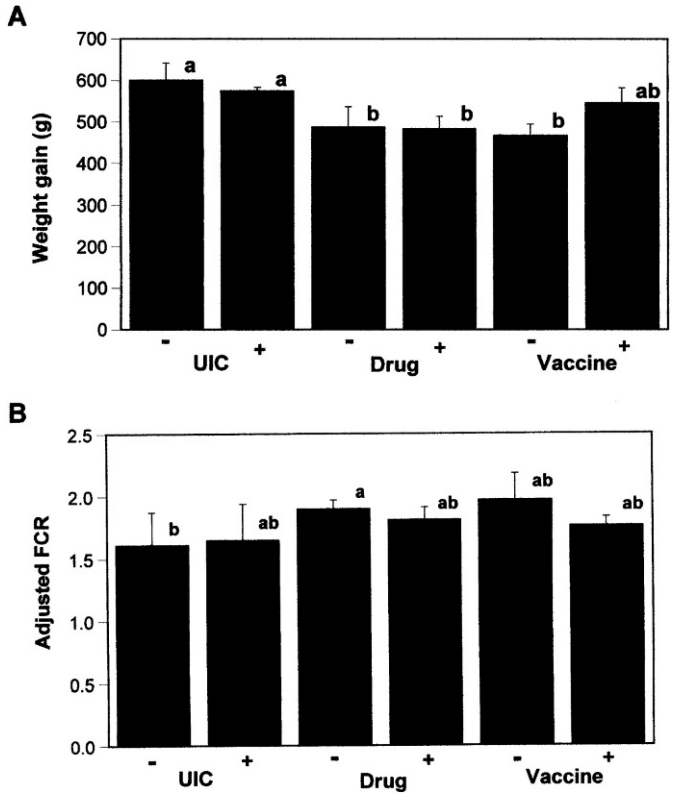

Fig. 2. Protective effect of salinomycin against decreased WG (A) and (B) adjusted feed conversion efficiency associated with coccidiosis in broilers infected with *Eimeria* oocysts isolated from ACD-utilizing or VAC-utilizing (Coccivac B) poultry operations in North Carolina. UIC, uninfected controls; −, groups given nonmedicated feed; +, groups given salinomycin (Biocox, 60 g/ton)-medicated feed. Groups sharing a letter do not have a significant difference ($P > 0.05$) in WG or adjusted FCR from each other.

oocysts/g, with no significant differences ($P > 0.05$) in oocyst concentration between ACD and VAC farms at any location (Table 1). PCR analysis revealed that *E. acervulina* and *E. maxima* were present in virtually all operations regardless of type of coccidiosis control (Table 1). *Eimeria tenella* was present in VAC-using farms, and absent in ACD-using farms in North Carolina and Delmarva (Table 1). Regarding the latter geographic location, *E. tenella* appeared in litter after the switch from salinomycin to vaccines (Table 1).

Comparison of salinomycin sensitivity between ACD-using and VAC-using poultry operations. In general, *Eimeria* spp. oocysts expanded from litter in VAC farms displayed greater sensitivity to salinomycin compared to ACD farms as measured by WG over the infection period. For instance, the average improvement in WG relative to the respective noninfected controls in broilers given salinomycin and infected with oocysts from ACD-using Arkansas farms was 12% greater than in broilers given unmedicated feed (Fig. 1A). However, an even greater improvement in WG (27% increase) was observed in broilers given salinomycin and infected with oocysts from VAC-using Arkansas farms (Fig. 1A). A similar increase in sensitivity was observed between ACD- and VAC-using North Carolina farms. The average improvement in WG in broilers given salinomycin and infected with oocysts from ACD-using farms was 3% compared to a 17% increase in average WG in broilers given salinomycin and infected with oocysts from VAC-using farms (Fig. 2A). Thus, broilers given salinomycin-medicated feed and infected with *Eimeria* oocysts from VAC farms showed a 15% greater improvement in WG compared to broilers raised on salinomycin and infected with *Eimeria* oocysts from ACD farms.

A significant improvement ($P < 0.05$) was also observed in adjusted FCR between nonmedicated and salinomycin-medicated chickens infected with either *Eimeria* oocysts from ACD-using or VAC-using Arkansas farms (Fig. 1B). Although not significant ($P > 0.05$), a measureable improvement was observed between feed conversion efficiency of broilers infected with oocysts from VAC-using compared to ACD-using North Carolina farms (Fig. 2B).

Comparison of salinomycin sensitivity after switching means of coccidiosis control. Greater salinomycin sensitivity was observed in *Eimeria* spp. oocysts expanded from VAC-using farms compared to oocysts collected from the same farms during ACD use. The average improvement in WG relative to the respective noninfected control in broilers given salinomycin and infected with oocysts from ACD-using Delmarva farms was 3% compared to WG improvement in broilers given unmedicated feed. However, even greater improvement in WG was observed in broilers given salinomycin and infected with oocysts from CoccivacB-using farms (25% WG increase) or with oocysts from Inovocox-using farms (33% WG increase).

A significant improvement ($P < 0.05$) in adjusted FCR was also observed between nonmedicated and salinomycin-medicated broilers infected with oocysts obtained during CoccivacB or Inovocox usage

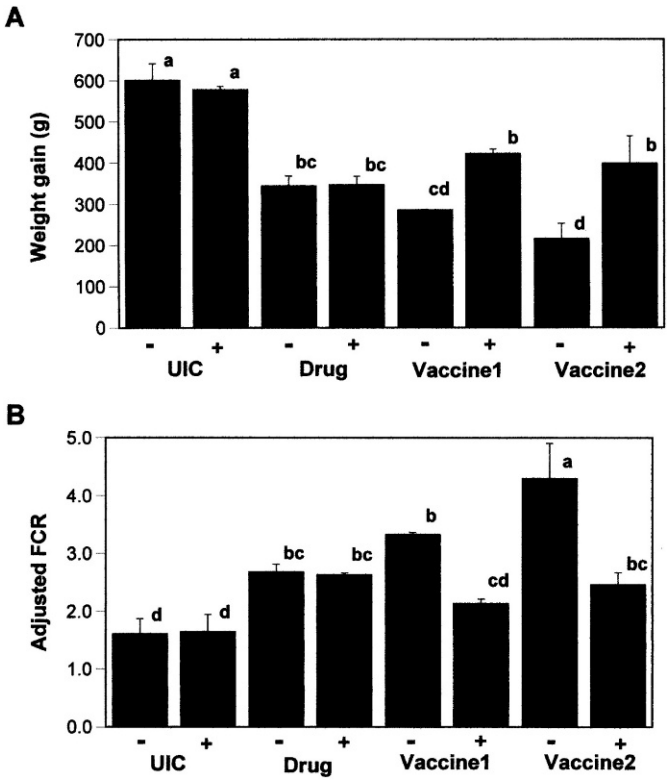

Fig. 3. Protective effect of salinomycin against (A) decreased WG and (B) adjusted feed conversion efficiency associated with coccidiosis in broilers infected with *Eimeria* oocysts isolated from ACD-utilizing or VAC-utilizing (vaccine 1, Coccivac B; vaccine 2, Inovocox) poultry operations in Delmarva (Delaware, Maryland, Virginia). UIC, uninfected controls; −, groups given nonmedicated feed; +, groups given salinomycin (Biocox, 60 g/ton)-medicated feed. Groups sharing a letter do not have a significant difference ($P > 0.05$) in WG or adjusted FCR from each other.

(Fig. 3B). In contrast, no significant improvement was observed between nonmedicated and salinomycin-medicated broilers infected with oocysts from the same operations during ACD usage (Fig. 3B).

DISCUSSION

In the present study, molecular techniques to identify *Eimeria* species present in poultry operations were used in conjunction with standard drug sensitivity testing to evaluate resistance of each *Eimeria* population to a commonly used ionophore—salinomycin. Our findings indicate that the *Eimeria* species population and sensitivity to salinomycin is affected by the particular coccidiosis control strategy being employed. For instance, in all three regions, salinomycin sensitivity was greater in *Eimeria* oocysts recovered from farms that were using VAC compared to farms using salinomycin. This is not surprising given that salinomycin resistance has a greater likelihood of occurring in *Eimeria* that are under drug pressure. Others have observed a similar phenomenon with synthetic drugs (decoquinate (9), diclazuril (13,18)) and an ionophore (monensin (18)). Although partial salinomycin resistance was observed in this study, it is probable that an accumulation of drug-resistant *Eimeria* takes a considerable length of time in a typical broiler operation. In our studies, repeated passages (>10) of *E. acervulina* in the presence of salinomycin failed to produce a drug-resistant strain of this coccidian (Jenkins, unpubl. data).

ACD- and VAC-using operations were similar in that *E. acervulina* and *E. maxima* were consistently found in both types of control strategies. However, for North Carolina and Delmarva operations, *E. tenella* was present on VAC-using farms and absent on ACD-using farms. It is possible that ACDs such as salinomycin prevented emergence of *E. tenella*, whereas VACs, because they contain *E. tenella*, introduce this species in poultry facilities. A consistent pattern between *Eimeria* oocysts recovered from VAC-using operations in all three regions was the greater salinomycin sensitivity compared to ACD-using farms as measured by improvement in WG. This confirms observations made by others on the capacity of VACs to replace or dilute the drug-resistant *Eimeria* in poultry facilities (2,13).

Another interesting phenomenon was the presence of *E. mitis* in litter from all VAC-using North Carolina farms. This is intriguing because this species is not present in the Coccivac B formulation, and affirms the value of molecular typing assays for *Eimeria* species that, for the most part, are indistinguishable from each other. As observed by our group and others, *E. mitis* and *E. praecox* were present on a subset of farms (11,17). In the future it may be advisable to incorporate these species in a vaccine formulation, especially given the recent description of a pathogenic *E. praecox* strain isolated from a commercial broiler operation (25).

REFERENCES

1. Allen, P., and R. Fetterer. Recent advances in biology and immunobiology of Eimeria species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin. Microbiol. Rev. 15:58–65. 2002.

2. Chapman, H. D., T. E. Cherry, H. D. Danforth, G. Richards, M. W. Shirley, and R. B. Williams. Sustainable coccidiosis control in poultry production: the role of live vaccines. Int. J. Parasitol. 32:617–629. 2002.

3. Chapman, H. D., B. Roberts, M. W. Shirley, and R. B. Williams. Guidelines for evaluating the efficacy and safety of live anticoccidial vaccines, and obtaining approval for their use in chickens and turkeys. Avian Pathol. 34:279–290. 2005.

4. Davis, L. R. Techniques. In: The coccidia. D. M. Hammond and P. L. Long, eds. University Park Press, Baltimore, MD. pp. 411–458. 1973.

5. Fernandez, S., A. H. Pagotto, M. M. Furtado, A. M. Katsuyama, A. M. Madeira, and A. Gruber. A multiplex PCR assay for the simultaneous detection and discrimination of the seven Eimeria species that infect domestic fowl. Parasitology 127:317–325. 2003.

6. Gasser, R. B., R. Skinner, R. Fadavi, G. Richards, and G. Morris. High-throughput capillary electrophoresis for the identification and differentiation of seven species of Eimeria from chickens. Electrophoresis 26:3479–3485. 2005.

7. Haug, A., P. Thebo, and J. G. Mattsson. A simplified protocol for molecular identification of *Eimeria* species in field samples. Vet. Parasitol. 146:35–45. 2007.

8. Holdsworth, P. A., D. P. Conway, M. E. McKenzie, A. D. Dayton, H. D. Chapman, G. F. Mathis, J. T. Skinner, H. C. Mundt, and R. B. Williams. World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anticoccidial drugs in chickens and turkeys. Vet. Parasitol. 121:189–212. 2004.

9. Jeffers, T. K. Reduction of anticoccidial drug resistance by massive introduction of drug-sensitive coccidian. Avian Dis. 20:649–653.

10. Jenkins, M. C., K. Miska, and S. Klopp. Application of polymerase chain reaction based on ITS1 rDNA to speciate Eimeria. Avian Dis. 50:110–114. 2006.

11. Jenkins, M. C., K. Miska, and S. Klopp. Improved polymerase chain reaction technique for determining the species composition of Eimeria in poultry litter. Avian Dis. 50:632–635. 2006.

12. Lew, A. E., G. R. Anderson, C. M. Minchin, P. J. Jeston, and W. K. Jorgensen. Inter- and intra-strain variation and PCR detection of the internal

transcribed spacer 1 (ITS-1) sequences of Australian isolates of Eimeria species from chickens. Vet. Parasitol. 112:33–50. 2003.

13. Mathis, G. F., and C. Broussard. Increased level of Eimeria sensitivity to diclazuril after using a live coccidial vaccine. Avian Dis. 50:321–324. 2006.

14. Morgan, J. A., G. M. Morris, B. M. Wlodek, R. Byrnes, M. Jenner, C. C. Constantinoiu, G. R. Anderson, A. E. Lew-Tabor, J. B. Molloy, R. B. Gasser, and W. K. Jorgensen. Real-time polymerase chain reaction (PCR) assays for the specific detection and quantification of seven Eimeria species that cause coccidiosis in chickens. Mol. Cell. Probes 23:83–89. 2009.

15. Morris, G. M., and R. B. Gasser. Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in Eimeria. Biotechnol. Adv. 24:590–603. 2006.

16. Morris, G. M., W. G. Woods, D. G. Richards, and R. B. Gasser. The application of a polymerase chain reaction (PCR)-based capillary electrophoretic technique provides detailed insights into Eimeria populations in intensive poultry establishments. Mol. Cell. Probes 21:288–294. 2007.

17. Morris, G. M., W. G. Woods, D. G. Richards, and R. B. Gasser. Investigating a persistent coccidiosis problem on a commercial broiler-breeder farm utilising PCR-coupled capillary electrophoresis. Parasitol. Res. 101:583–589. 2007.

18. Peek, H. W., and W. J. Landman. Higher incidence of Eimeria spp. field isolates sensitive for diclazuril and monensin associated with the use of live coccidiosis vaccination with paracox-5 in broiler farms. Avian Dis. 50:434–439. 2006.

19. Ryley, J. F., R. Meade, J. Hazelhurst, and T. E. Robinson. Methods in coccidiosis research: separation of oocysts from faeces. Parasitology 73:311–326. 1976.

20. Schnitzler, B. E., P. L. Thebo, F. M. Tomley, A. Uggla, and M. W. Shirley. PCR identification of chicken Eimeria: a simplified read-out. Avian Pathol. 28:89–93. 1999.

21. Shirley, M. W., A. L. Smith, and D. P. Blake. Challenges in the successful control of the avian coccidia. Vaccine 25:5540–5547. 2007.

22. Sun, X. M., W. Pang, T. Jia, W. C. Yan, G. He, L. L. Hao, M. Bentué, and X. Suo. Prevalence of Eimeria species in broilers with subclinical signs from fifty farms. Avian Dis. 53:301–305. 2009.

23. Williams, R. B. Fifty years of anticoccidial vaccines for poultry (1952–2002). Avian Dis. 46:775–802. 2002.

24. Williams, R. B. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathol. 31:317–353. 2002.

25. Williams, R. B., R. N. Marshall, M. Pagés, M. Dardi, and E. del Cacho. Pathogenesis of Eimeria praecox in chickens: virulence of field strains compared with laboratory strains of E. praecox and Eimeria acervulina. Avian Pathol. 38:359–366. 2009.